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(54) Title: ANTIBIOTIC-METAL COMPLEX AND METHODS

(57) Abstract: A complex formed from a cyclic antibiotic and a metal is useful for detecting gram negative bacteria and also has medical and veterinary diagnostic and therapeutic applications.

ANTIBIOTIC-METAL COMPLEX AND METHODS

This application claims the benefit of U.S. Provisional Application Serial No. 60/159,142, filed October 13, 1999, which is incorporated herein by reference in its entirety.

Background of the Invention

The term "coliform bacteria" as used herein refers to a group of bacterial genera made up of *Escherichia, Klebsiella, Enterobacter, Serratia* and *Citrobacter* bacteria. Coliform bacteria tend to be small, gram negative rods that may be either motile or nonmotile. Coliform bacteria have complex membranes that include murein, lipoprotein, phospholipid and lipopolysaccharide (LPS) components arranged in layers. A murein-LPS layer is about 20% of the total bacterial membrane and is responsible for bacterial cell rigidity. The LPS aids in preventing hydrophobic toxins from entering a coliform bacterial cell. The LPS is capable of releasing an endotoxin into a host once coliform bacteria infect the host. In human hosts, the endotoxin is released into the bloodstream.

Natural competitors of coliform bacteria have evolved secondary metabolites, such as antibiotics, to overcome the LPS defense. Competitors such as soil fungi and *Streptomycetes* as well as gram positive bacteria produce antibiotics. One particular class of lipopeptide antibiotic, polymyxins, are produced by a soil microorganism, *B. polymyxa*.

The polymyxins are designated by the letters A, B, C, D and E. The polymyxins are toxic to coliform bacteria because these antibiotics bind to the LPS in an outer membrane of the coliform bacteria and disrupt cellular metabolism of a coliform bacterium once translocated to an inner cytoplasmic membrane. In particular, the polymyxins are believed to alter the structure and osmotic properties of the outer membrane. Intact, polymyxin antibiotics are capable of binding to both animal membranes and coliform bacterial

membranes.

Coliform bacteria as well as some fungi may cause infections in the urinary tract and wounds of a human host. Coliform bacteria may also cause pneumonia, meningitis, septicemia and various gastrointestinal disorders in a human being. It has been estimated that as many as 100,000 deaths in the United States each year are a consequence of gram negative bacteria infections such as coliform bacteria.

In addition to causing disease by direct infection, the endotoxin produced by coliform bacteria produces a variety of effects, such as fever, fatal shock, leukocytic alterations, cytotoxicity, alterations in host response to infections, Sanarelli-Shwartzman reaction and various other undesirable metabolic changes. When coliform bacteria enter the bloodstream of a human being, endotoxic shock plays an important role in weakening the individual. About 30% of individuals with endotoxin in their blood will develop shock. About 40 to 90% of individuals in endotoxic shock die. Endotoxin shock is characterized by an inadequacy of blood supply to vital organs of the host causing cellular hypoxia and metabolic failure. Survival of the host is directly proportional to the length of time needed to recognize the development of bacteremia and adequate treatment of the coliform bacterial infection.

Unfortunately, to date, testing for coliform bacteria, yeast and fungi has been excessively time consuming and labor intensive. While the onset of symptoms from the endotoxin may be exceedingly rapid, laboratory based diagnosis will typically take days. To detect and identify coliform bacteria, it is necessary to expose suspect specimens, such as sputum, tissue, pus, body fluids, rectal swabs or feces to a culture media that will allow the growth of gram negative bacteria but inhibit the growth of gram positive bacteria.

The present techniques used for this type of screening involve aseptic transfer of a sample, streaking the sample having bacterial organisms on agar plates after serial dilution and colony enumeration. This is a laborious and lengthy process requiring a time of at least about 24 to 48 hours for a positive result and substantially longer for a negative result.

Additionally, test solutions containing enteric bacteria use carbohydrates and acid based indicators to demonstrate carbohydrate fermentation. Lactose and analogues of lactose are the carbohydrates most frequently used in bacteria testing. This is because the majority of organisms of the genera *Escherichia*, *Enterobacter*, and *Klebsiella*, the enteric organisms present in greatest number in fecal material, ferment this carbohydrate while other intestinal pathogens usually do not. Some media may also contain iron salts for the detection of hydrogen sulfide production to aid in the identification of *Salmonella* colonies. This approach to bacterial testing also requires a lengthy incubation time to grow enough bacteria for testing, at least 24 to 48 hours.

Other analyte tests require an organism to digest a detectable material such as fluorescein. In other tests, an antibody, specific for an antigen on an analyte, is labeled with fluorescein to make a fluorescent antibody. Another approach involves the use of a visualization polymer coupled to a detecting agent that binds the target organism, wherein the visualization polymer is made up of detectable visualization units, such as multiple enzymes or labeled polyolefins, which are directly or indirectly bonded together (Ward et al. U.S. Pat. No. 4,687,732). Another approach involves covalent conjugation of polymyxin B (PMB) and an enzyme reporter molecule, such as horseradish peroxidase (HRP), to produce a complex for use in a binding assay to detect the target organism (Appelmelk et al., Anal. Biochem. 207:311-316 (1992)). An organic "chemical tag" that comprises populations of binding agents and detectable labels has also been described (Olstein et al., U.S. pat. No. 5,750,357).

However, a continuing need exists for a sensitive and rapid method to detect extremely small amounts of target biological analytes.

Summary of the Invention

The present invention provides a molecular complex, preferably a chelated complex, formed from an antibiotic, preferably a cyclic antibiotic such as a polymyxin, and a metal, preferably a transition metal or lanthanide metal. The complex preferably has one or more optical properties (e.g., fluorescence, UV or visible light absorbance, or paramagnetic character) that allows it to be easily detected. In one aspect, the complex binds to and thereby allows detection of gram negative bacteria, for example in applications related to food processing or medical sterilization. In another aspect, the complex is attached to a monoclonal antibody or other delivery/carrier molecule, and can function as a targeted detection agent and/or therapeutic agent in medical or veterinary applications. It should be understood that the invention includes the antibiotic-metal complex, methods of making the antibiotic-metal complex, and methods of using the antibiotic-metal complex.

Brief Description of the Drawings

Figure 1 depicts the MM+ energy minimized model of the polymyxin B structure rendered in a molecular modeling package available from HALLoGRAM Publishing (Aurora, CO) under the tradename HYPERCHEM.

Figure 2 depicts the UV-visible spectrum of the ferric complex of polymyxin B.

Figure 3 depicts the UV-visible spectrum of the cobalt complex of polymyxin B.

Figure 4 depicts the UV-visible spectrum of the copper(II) complex of polymyxin B.

Figure 5 depicts the overlay UV-visible spectrum of the terbium complex of polymyxin B, the ferric complex of polymyxin B, and the copper complex of polymyxin B.

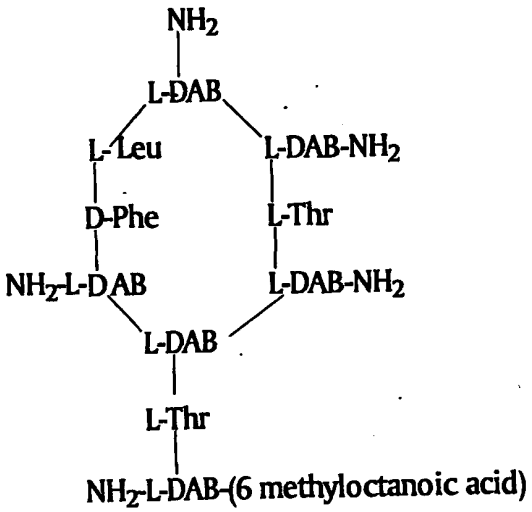
Figure 6 depicts a titration curve, using the cobalt complex of polymyxin B, for *E. coli* O157:H7.

Figure 7 depicts a titration curve, using the cobalt complex of polymyxin B, for *Salmonella enteritis*.

Figure 8 depicts a cell dilution curve, using the cobalt complex of polymyxin B, for *Heliobacter pylori*.

Detailed Description of the Preferred Embodiments

Polymyxin B, like the other polymyxins, is a cyclic decapeptide having a high percentage of 2,4-diaminobutyric acid (Dab), a fatty acid and a mixture of D- and L-amino acids. Polymyxin B has the following structure:



A preferred molecular complex of the invention takes the form of an isolated, bound complex comprising a cyclic antibiotic and a metal. The antibiotic is preferably a polymyxin or a colistin. The polymyxin or colistin can be a decapeptide or a peptide subunit or fragment thereof, preferably a nonapeptide. Examples of polymyxin subunits or fragments are described, for example, in R. L. Danner et al., Antimicrob. Agents Chemother., 33:1428-1434 (1989); M. Vaara et al., Antimicrob. Agents Chemother. 24:107-113 (1983); and

U.S. Pat. No. 5,750,357 (Olstein et al.). A preferred polymyxin or colistin peptide fragment is one that retains bacterial binding activity and is preferably less toxic to mammalian tissues than naturally occurring polymyxins. Preferably, the metal in the complex is a transition metal or a lanthanide metal; more preferably it is copper, cobalt, iron (Fe^{2+} and Fe^{3+}), gadolinium, europium, terbium or technicium. Preferably, the polymyxin is polymyxin B (PMB). The term "polymyxin-metal complex", as used herein, is intended to encompass all the above-identified embodiments of the complex.

The polymyxin-metal complex preferably comprises at least one molecule of polymyxin and at least one atom of the metal. In a particularly preferred embodiment, there is a 1:1 stoichiometry between the metal and the cyclic peptide. Experimental results (described below) indicate that the polymyxin-metal complex of the invention can be a chelated metal complex. The invention, however, is not limited to complexes with chelation interactions but encompasses, for example, polymyxin-metal complexes formed from covalent or electrostatic interactions. In a chelated complex, the metal can be coordinated at more than one site, preferably at four, five, or six sites. The metal binding site may be within a cleft formed by the cyclized amino acids. A cleft is shown below in Fig. 1 in a MM+ energy minimized model of the polymyxin B structure rendered in a commercial molecular modeling package, HYPERCHEM.

The polymyxin-metal complex of the invention is unique in that it allows detection of gram negative bacteria, preferably pathogenic bacteria, without covalently linking the polymyxin to a detectable label. Polymyxin-metal complexes can directly catalyze peroxide-driven chemiluminescent reactions (for example, reactions involving luminol, its aromatic heterocyclic derivatives, lucigenin, penicillin, luciferin, and other polyaromatic phthalyhydrazides; see, e.g., M. Rost et al., J. Biolumin. Chemilumin. 13:355-363 (1998)) without the use of an intermediate such as horseradish peroxidase (HRP) or

microperoxidase. Moreover, while most organic derivatives of polymyxin exhibit a loss of bioactivity (i.e., toxicity or bacteriocidal activity) compared to underivatized polymyxin, the polymyxin-metal complexes appear to retain bioactivity. These attributes make the polymyxin-metal complexes better diagnostic tools than previously known PMB-HRP conjugates.

Many of these complexes have optical properties (e.g., fluorescence, UV or visible light absorbance, or paramagnetic character) that allow them to be easily detected. For example, PMB-metal complexes with Tb and Eu fluoresce, and PMB-metal complexes with iron and cobalt are colored, allowing direct visualization of the complex.

The polymyxin-metal complex can be readily purified using standard chromatrographic techniques such as gel filtration because it can be followed visually or using fluorescence or UV detection, depending on the type of complex. Immobilized LPS also has been shown to bind PMB-metal complexes of the invention. Although the complex appears to be stable at room temperature, it is preferably refrigerated and stored in the dark.

The invention further provides a method for detecting gram negative bacteria, preferably pathogenic gram negative bacteria, comprising adding a polymyxin-metal complex to a sample suspected of containing gram negative bacteria, washing away the unbound complex, adding a chemiluminescent agent such as luminol or lucigenin, then measuring the resulting luminescence using a luminometer.

After synthesis, the polymyxin-metal complexes of the invention can be freeze-dried or spray-dried and is preferably stored in the dark, then reconstituted in water or an appropriate buffer for later use.

The polymyxin-metal complexes of the invention are capable of numerous and varied diagnostic and therapeutic uses. For example, gram negative bacteria can be rapidly detected in any environment, such as part of quality control efforts in food processing and sterilization of medical devices or medical fluids, such as pharmaceutical carriers or vehicles, saline solutions or nutritional fluids for parenteral administration. Preferably, the polymyxin-metal complex has a lower limit of detection of gram negative bacteria of about 10

CFU/mL or less. Paramagnetic polymyxin-metal complexes, such as those comprising Gd, can used in medical magnetic resonance imaging (MRI) to detect infection, disease, or autoimmune dysfunction by attaching the complex to a monoclonal antibody (MAb) raised against the cell to be detected. For example, the polymyxin-metal complex could be mono-thiolated using a reagent such as N-acetyl homocysteine thiolactone or iminothiolane. The antibodies could then be crosslinked to the polymyxin-metal complexes using any of a variety of maleimide derivatizing agents to form a molecular complex containing a polymyxin, a metal, and an antibody. Therapeutic use of a polymyxin-metal-MAb complex is also envisioned, either as a targeted bifunctional imaging/therapeutic agent or as a purely therapeutic agent. Complexes of polymyxin with radioactive Tc (^{99}Tc) attached to a MAb or other delivery/carrier molecule also have potential as targeted therapeutic agents. These diagnostic and therapeutic uses have great promise in the fields of cancer and AIDS treatment. For example, a therapeutic complex such as a polymyxin-^{99}Tc-MAb complex could be targeted to a cancer cell and function as both a therapeutic and imaging agent. Peptide-metal complexes like the polymyxin-metal complexes of the present invention are preferred over protein-metal complexes for these uses because they are less likely to be involved in nonspecific interactions, thereby reducing detection background levels.

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

EXAMPLES

Example I. Organic Polymyxin Derivatives

As described in the Background section, above, attempts have previously been made to develop organic antibiotic-enzyme conjugates for detection of bacteria by introducing a reporter group onto the antibiotic without compromising the binding and anti-microbial activity of the conjugate. However, organic derivatization of antibiotics has disadvantages.

First, organic derivatives of the antibiotic lose appreciable anti-microbial activity, as well as binding activity. Table 1 shows bioactivity for PMB and selected PMB derivatives. Standard minimum inhibitory concentrations (MIC) were determined for the indicated polymyxin B derivatives to quantitate loss of biological activity. Data were acquired using *E. coli* grown in liquid culture containing tryptic soy broth (TSB) culture medium (Millipore Corporation).

Table 1. Bioassay for Polymyxin Derivatives

Comp	10γ	9γ	8γ	7γ	6γ	5γ	4γ	3γ	2γ	1γ	0.13γ
PMB	-	-	-	-	-	-	-	-	-	-	+
PMB-HcTl	-	-	-	+	+	+	+	+	+	+	ND
Glyc-PMB	+	+	+	+	+	+	+	+	+	+	ND
Imthio-PMB	+	+	+	+	+	+	+	+	+	+	ND
ThRAl-PMB	+	+	+	+	+	+	+	+	+	+	ND

PMB = polymyxin B pentasulfate

PMB-HcTl = polymyxin B modified with N-acetyl homocysteine thiolactone

Glyc-PMB = polymyxin B modified with mannose by reductive alkylation

ThRAl-PMB = polymyxin B thiolated on solid phase by reductive alkylation

Imthio-PMB = polymyxin B modified with iminothiolane

γ = micrograms/milliliter (μg/mL); + indicates growth; - indicates no growth

Second, the conjugates prepared to date have inordinately high non-specific adsorption characteristics to material surfaces, which renders the probe less useful for sensitive analytical measurement.

Example II. Polymyxin-Metal Complexes

To our surprise, we have found that the polymyxin B molecule appears to bind transition metals and lanthanide metals very tightly.

Figure 1 depicts the MM+ energy minimized model of the polymyxin B structure rendered in a commercial molecular modeling package, HYPERCHEM. The cyclodecapeptide molecule exhibits a cleft in the molecule. Without intending the invention to be limited by any proposed theory of operability, there may be sufficient orbital overlap of the metal d orbitals with non-bonded electrons from either the amide nitrogens or the carbonyl oxygens to form metal-organic ligands to permit metal ion chelation. Several peptides have been documented which exhibit this activity, such as the ionophores valinomycin (Duax et al., Science, 176:911 (1972)) and antamanide (Y. Ovchinikov, Mitochondria Biomembr. Proc. Fed. Eur. Biochem. Soc., 28:279 (1972)). Polymyxin B has not been documented to function as an ionophore, which it probably does not. Unlike the ionophores, it does not exhibit selectivity for specific metal ions, but appears to be able to chelate a range of metals. The following examples speak to the issue of chelation versus electrostatic binding.

The selected metals have been documented to be very active in catalyzing hydrogen peroxide oxidation of luminol (W. Rost et al., J. Biolumin. Chemilumin., 13:355-363 (1998)). Thus, it is expected that these polymyxin B-metal complexes will bind to gram negative bacteria since they are biologically active (see Table 2) and also bind to immobilized lipopolysaccharide (on Sepharose 6B).

A. F rmation of a polymyxin B-terbium complex

Polymyxin B pentasulfate (Sigma) (80 milligrams (mg), 0.05 millimoles (mmol)), was dissolved in 5 mL of 0.05 M acetate buffer, pH 5.5. The peptide was treated with terbium trichloride, (21 mg, 0.055 mmol) (Molecular Probes, Inc.). This lanthanide metal is non-fluorescent in solution, however, if it is chelated, such as in the presence of EDTA or dipicolinic acid, it fluoresces with a blue emission. The complex was purified by chromatography on Sephadex G-25 column 2.5 x 12 cm. The complex exhibited a blue fluorescent emission, when illuminated with 330 nm light, which co-eluted with the peptide from the column.

B. Formation of a polymyxin B-iron (III) complex

Polymyxin B pentasulfate (Sigma) (80 mg, 0.05 mmol) was dissolved in 5 mL of 0.1 M acetic acid and treated with ferric chloride (15 mg, 0.055 mmol). After brief incubation at room temperature, the preparation was gel filtered on a Sephadex G-25 column, 4.5 x 10 cm. The A_{420}-absorbing material co-eluted with the UV absorbing material. These fractions were pooled and freeze dried. The freeze dried material exhibited anti-microbial activity of 2.5 μg/mL in the minimum inhibitory concentration assay. The UV-visible spectrum, Figure 2, of the isolated peptide exhibits characteristic soret bands for planarly coordinated iron ligands such as those seen in porphyrin compounds.

C. Formation of a polymyxin B-cobalt complex

Polymyxin B pentasulfate (80 mg, 0.05 mmol) was dissolved in 5 mL 0.05 M acetate buffer, pH 5.5. The antibiotic was treated with cobalt chloride (12 mg, 0.055 mmol), incubated at room temperature briefly, and purified by chromatography on a Sephadex G-25 column as in Example IV. The UV-absorbing fractions were collected and freeze dried. The UV-visible spectrum, Figure 3, shows a weak absorbance in the visible-near UV region centered at 340 nm.

D. Formation of a polymyxin B-copper (II) complex

Polymyxin B pentasulfate (80 mg, 0.05 mmol) was dissolved in 5 mL of 0.1 M acetic acid. $Cu(NO_3)_2$ (12 mg) was dissolved in 0.2 mL of buffer and added with stirring at room temperature. After ten minutes, the complex was purified by chromatography on a Sephadex G-25 column. The blue copper containing band co-eluted with the UV-absorbing peptide fractions. The material was freeze dried and an electronic spectrum, Figure 4, was obtained.

E. Comparative spectral analysis

The individual spectra reveal evidence of the visible absorbance bands, however, the overlay spectra, Figure 5, demonstrate the dramatic effect the metal complexes have on the intensities optical properties of these compounds. The lanthanide complex (containing terbium) is entirely transparent in the visible and to a greater extent the near UV as well. The data closely parallel the spectrum for the un-complexed antibiotic. The transition metal complexes (containing iron, cobalt and copper) absorb more intensely in the near UV and the blue end of the visible spectrum where characteristic Soret bands are observed for porphyrin complexes of iron and copper.

F. Comparative bioactivity

Table 2 shows bioactivity for PMB and selected PMB-metal complexes. Standard minimum inhibitory concentrations (MIC) were determined for the indicated polymyxin B complexes to quantitate loss of biological activity. Data were acquired using *E. coli* grown in liquid culture containing TSB medium.

Table 2. Bioassay for Polymyxin-Metal Complexes

Cmp.	10γ	9γ	8γ	7γ	6γ	5γ	4γ	3γ	2γ	1γ
PMB	-	-	-	-	-	-	-	-	-	-
CoPMB	-	-	-	-	-	-	-	-	-	+
TbPMB	-	-	-	-	-	-	-	-	-	+
CuPMB	+	+	+	+	+	+	+	+	+	+
MnPMB	-	-	-	-	-	-	-	+	+	+

γ = μg/mL; + indicates growth; - indicates no growth

G. Polymyxin B fragments or subunits

The use of polymyxin B nonapeptide (R. L. Danner et al., Antimicrob. Agents Chemother., 33:1428-1434 (1989); M. Vaara et al., Antimicrob. Agents Chemother. 24:107-113 (1983)) as a metal ligand is also contemplated since the binding activity does not appear to be compromised and the cleft is still left in the molecule after proteolytic digestion by ficin (M. Vaara et al., Antimicrob. Agents Chemother. 24:107-113 (1983); M. Vaara t al., Antimicrob. Agents Chemother., 24:114-122 (1983)). See also U.S. Pat. No. 5,750,357 (Olstein et al.).

Example III. Binding Activity of Polymyxin B-Iron (II) Complex to Immobilized Lipopolysaccharide

Lipopolysaccharide resin was prepared as follows. Epoxy-activated Sepharose 4B (Sigma) (5 g) was washed with distilled water and subsequently dehydrated with water-ethanol 20:80 (volume/volume (v/v)), water-ethanol 50:50 (v/v), and ethanol. Purified lipopolysaccharide (LPS) from *Salmonella enteridis* (Sigma) (10 mg) was dissolved in 18 mL of dimethyl sulfoxide. The dehydrated epoxy resin was suspended in the LPS solution and 0.13 mL of tributylamine was added. The suspension was agitated eighteen hours at room temperature. Unbound epoxy groups were blocked by addition of 5 mL of 0.2 M glucosamine, free base, water:DMSO and incubated a further 24 hours at room temperature. The resin was progressively washed with solvents to re-

suspend in 100% aqueous medium.

Five mL of 2 mg/mL solution of the iron (II) polymyxin B complex in 0.05 M acetate buffer, pH 5.5, was applied to a 2 mL LPS-resin column. All of the colored metal complex and the UV absorbing peptide was strongly bound to the resin. This data demonstrates that the complex still maintains LPS binding competency.

Example IV.

Quantitation of Gram Negative Organisms with Polymyxin B-Cobalt Complex.

A. Cell titration of *E. coli* O157:H7

Bacteria were diluted in sterile saline from cell concentrations of 10^8 CFU/mL to 10 CFU/mL. The cells were treated with the PMB-Co(II) complex at 20 μg/mL for twenty minutes at room temperature. The cells were centrifuged, rinsed with 1.0 mL saline; centrifuged and re-suspended in 0.1 mL saline. Chemiluminescence was measured using 0.2 mL of Luminol reagent purchased from NEN Life Sciences (Boston, MA) in a luminometer available from Biotrace (UK). Figure 6 shows the titration curve for the cells.

B. Cell titration of *Salmonella enteritis* FDA strain

Bacteria were diluted in sterile saline from cell concentrations of 10^8 CFU/mL to 10 CFU/mL. The cells were treated with the PMB-Co(II) complex at 20 μg/mL for twenty minutes at room temperature. The cells were centrifuged, rinsed with 1.0 mL saline; centrifuged and re-suspended in 0.1 mL saline. Chemiluminescence was measured using 0.2 mL of Luminol reagent purchased from NEN Life Sciences in a Biotrace luminometer. Figure 7 shows the titration curve for the cells.

C. Quantitation f *Heliobacter pylori*

In vitro cultivation of *H. pylori* is difficult because the organism does not readily grow on media developed for solid culture; consequently, methods for

bacterial enumeration are not readily available. However, we were able to show a linear response of this binding assay in response to cell dilution in liquid medium, Figure 8.

D. Negative response from *Listeria monocytogenes*

L. monocytogenes is a gram positive organism. Thus, one would expect a negative response from this test since the antibiotic does not bind to gram positive cells. Bacteria were diluted in sterile saline from cell concentrations of 10^8 CFU/mL to 10 CFU/mL. The cells were treated with the PMB-Co(II) complex at 20 μg/mL for twenty minutes at room temperature. The cells were centrifuged, rinsed with 1.0 mL saline; centrifuged and re-suspended in 0.1 mL saline. Chemiluminescence was measured using 0.2 mL of Luminol reagent purchased from NEN Life Sciences in a Biotrace luminometer. The cells exhibited background levels of chemiluminescence at all cell concentrations tested.

E. Linearity of titration response curve

In some instances, linearity of the titration response curve may not be observed for the entire range of cell concentrations. This may be due to a propensity of cells to clomp or aggregate, preventing the cells from behaving as finely dispersed particles in a suspension. In such cases, cells could be detected but not readily quantified. The problem could be obviated by use of a plaque lift assay coupled with plating on medium to determine if cells spread on solid medium could be seen after a few minutes by labeling and imaging with a CCD camera, since the probe should be catalyzing sufficient photons to image. This assay could be done, for example, using a film available under the tradename PETRIFILM from 3M Company (Maplewood, MN).

Example V. Detection of *E. coli* in Food Sample

Immunomagnetic capture technique for the separation of bacteria from ground beef samples is described in Pyle et al.(Appl. Environ. Microbiol., 65:1966-1972 (1999)). Samples are processed as follows:

A one gram sample of ground beef is weighed into a Stomacher '80' bag (Seward) and 0.1 mL of an *E. coli* O157 (10^1 to 10^7 CFU/mL) suspension is inoculated into the sample. The meat and sample are massaged in the bag for 5 minutes. Twenty mL of 0.1% of peptone media is added before the bag is heat sealed. The sample is then homogenized in a Stomacher (Seward) for 2 minutes. The bag is opened aseptically and 20 mL of an enzyme-detergent solution (U. M. Rodrigues-Szulc et al., J. Appl. Bacteriol., 80:673-681 (1996)) is added. The bag is resealed and agitated on an orbital shaker for 20 minutes at room temperature. The contents are homogenized in the Stomacher a further 2 minutes, and the bag opened and the contents are passed through a course 25 mm screen to remove fibrous material. The sample is then inoculate with 0.01 mL of immuno-magnetic bead suspension (Dynal, Finland) coated with antibody to *E. coli* O157:H7 per mL of sample. After 60 minute incubation at room temperature, the paramagnetic particles are collected by placement of the vials in a supermagnetic particle concentrator (Dynal) for 30 minutes. The supernatant is then carefully removed by aspiration.

The collected beads bearing *E. coli* cells are then re-suspended in 1.0 mL saline and treated with 20 µg/mL of the polymyxin B-cobalt(II) complex for 10 minutes. The cells are collected in the particle concentrator and re-suspended in 0.1 mL and assayed for chemiluminescent oxidation of luminol, as previously described.

The complete disclosure of all patents, patent applications and publications cited herein are incorporated by reference. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claim.

WHAT IS CLAIMED IS:

1. A polymyxin-metal complex.

2. The polymyxin-metal complex of claim 1 wherein the polymyxin is selected from the group consisting of polymyxin B and colistin.

3. Diagnostic or therapeutic use of the polymyxin-metal complex of claim 1.

4. Methods of making the polymyxin-metal complex of claim 1.

5. A complex comprising a cyclic antibiotic and at least one of a lanthanide or a transition metal.

6. The complex of claim 5 wherein the cyclic antibiotic is a polymyxin or a subunit or fragment thereof.

7. The complex of claim 5 wherein the cyclic antibiotic is polymyxin B.

8. The complex of claim 5 wherein the cyclic antibiotic is colistin.

9. The complex of claim 5 wherein the cyclic antibiotic is a nonapeptide subunit of polymyxin B.

10. The complex of claim 5 wherein the metal is selected from the group consisting of copper, cobalt, iron(II), iron(III), gadolinium, europium, terbium or technicium.

11. The complex of claim 5 which possesses at least one detectable property.

12. The complex of claim 11 wherein the detectable property is selected from the group consisting of fluorescence, UV absorbance, visible light absorbance and paramagnetism.

13. The complex of claim 11 which catalyzes a chemiluminescent reaction.

14. The complex of claim 13 which catalyzes a chemiluminescent reaction involving a polyaromatic phthalyhydrazide.

15. The complex of claim 13 which catalyzes a chemilumiscent reaction involving a substance selected from the group consisting of luminol, its aromatic heterocyclic derivatives, lucigenin, penicillin and luciferin..

16. The complex of claim 5 further comprising a monoclonal antibody.

17. A method for detecting gram negative bacteria in a sample suspected of containing gram negative bacteria comprising:

contacting the sample with the complex of claim 11 such that the complex binds to the gram negative bacteria to yield a bound complex;

separating the bound complex from any nonbound complex; and

detecting the presence or absence of a bound complex, wherein the presence of a bound complex is indicative of the presence of gram negative bacteria.

18. The method of claim 17 wherein the sample is selected from the group consisting of a food sample, a medical sample and a biological sample.

19. The method of claim 18 wherein the medical sample is a medical device or medical fluid.

20. The method of claim 18 wherein the biological sample is a bodily tissue, organ or fluid.

21. The method of claim 17 wherein the antibiotic is polymyxin B.

22. A method for detecting disease in a patient suspected of having the disease, the method comprising:

introducing a detectable complex comprising a cyclic antibiotic, a metal and a delivery molecule into the patient, wherein the delivery molecule targets the complex to a disease cell, if present; and

detecting the presence or absence of the complex at a site within the patient, wherein the presence of the complex at the site is indicative of the presence of a disease cell in the patient at the site.

23. The method of claim 22 wherein the delivery molecule is a monoclonal antibody.

24. The method of claim 22 wherein the metal is a paramagnetic metal.

25. The method of claim 24 wherein the paramagnetic metal is gadolinium (Gd).

26. The method of claim 24 wherein detecting the complex comprises using magnetic resonance imaging (MRI).

27. The method of claim 22 wherein the detectable complex has a therapeutic effect on the patient.

28. The method of claim 27 wherein the metal comprises a radioactive metal.

29. The method of claim 28 wherein the radioactive metal comprises radioactive technicium (^{99}Tc).

30. The method of claim 22 wherein the disease cell is a bacterial cell, a cancer cell, or a cell involved in autoimmune dysfunction.

31. The method of claim 22 wherein the antibiotic is polymyxin B.

32. A method for detecting the presence of gram negative bacteria in a patient suspected of comprising gram negative bacteria, the method comprising:

introducing a detectable complex comprising a cyclic antibiotic and a metal into the patient; and

detecting the presence or absence of the complex at a site within the patient, wherein the presence of the complex at the site is indicative of the presence of gram negative bacteria in the patient at the site.

33. The method of claim 32 wherein the detectable complex kills or disables the gram negative bacteria.

34. The method of claim 32 wherein the antibiotic is polymyxin B.

35. A method for treating infection, disease or autoimmune dysfunction in a patient, the method comprising introducing a therapeutic complex comprising a cyclic antibiotic, a metal and a delivery molecule into the patient, wherein the delivery molecule targets the complex to a disease cell, if present.

36. The method of claim 35 wherein the disease cell is a bacterial cell, a cancer cell, or a cell involved in autoimmune dysfunction.

37. The method of claim 35 wherein the metal comprises a radioactive metal.

38. The method of claim 37 wherein the radioactive metal comprises radioactive technicium (^{99}Tc).

39. The method of claim 35 wherein the antibiotic is polymyxin B.

40. A method for detecting gram negative bacteria in a food sample comprising:

incubating the sample with immunomagnetic beads coated with antibody to the gram negative bacterium such that gram negative bacteria bind to the immunomagnetic beads;

magnetically removing the immunomagnetic beads from the sample;

contacting the immunomagnetic beads with the detectable complex of claim 11 to yield a detectable bound complex; and

assaying the immunomagnetic beads for the presence or absence of detectable bound complex, wherein the presence of a detectable bound complex is indicative of the presence of gram negative bacteria in the food sample.

41. The method of claim 40 wherein the antibiotic is polymyxin B and the assay is a chemiluminescent assay.

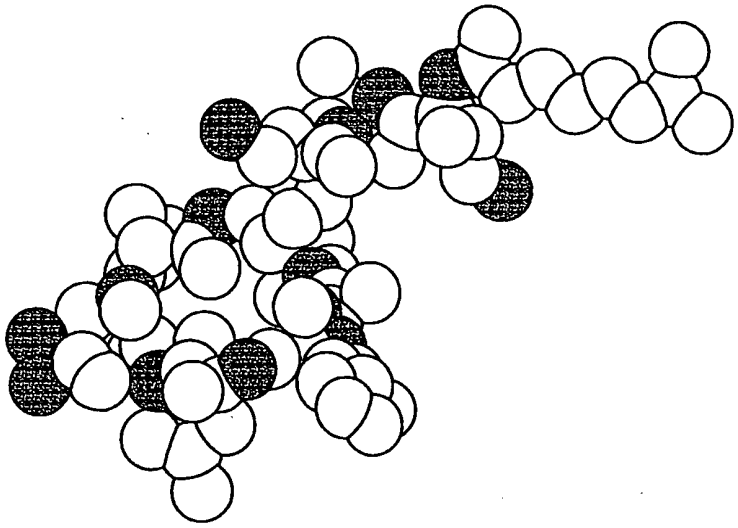


Fig. 1

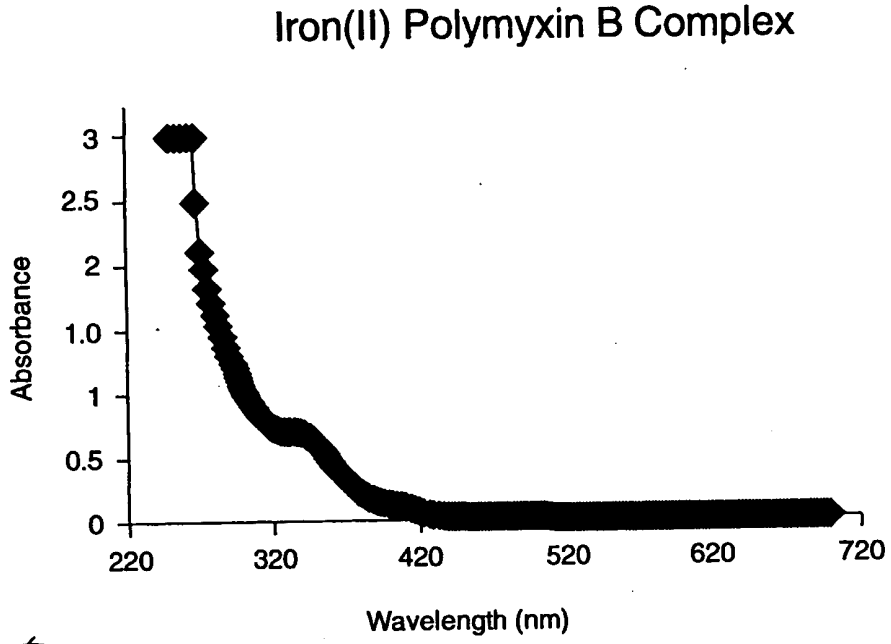

Iron(II) Polymyxin B Complex
Absorbance
3
2.5
2
1.0
1
0.5
0
220
320
420
520
620
720
Wavelength (nm)

Fig. 2

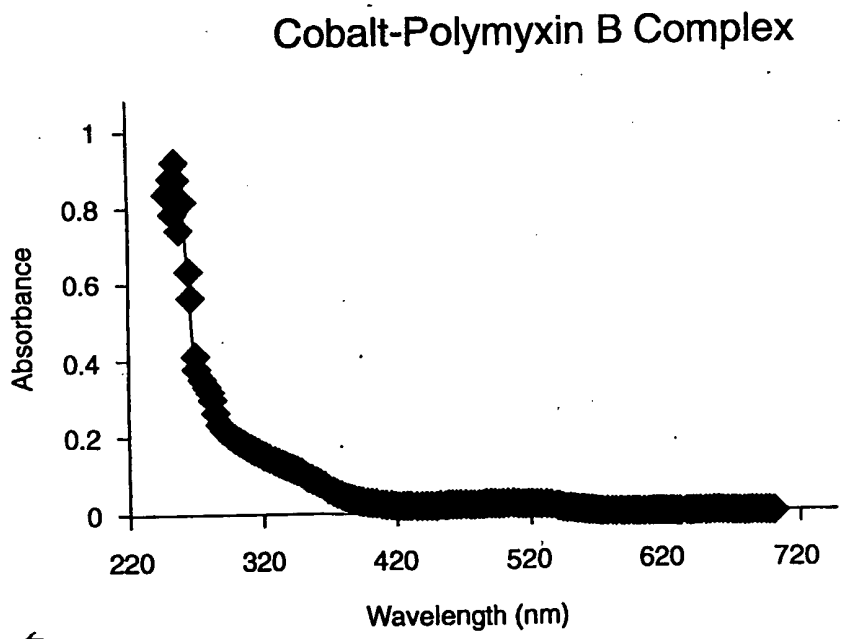

Cobalt-Polymyxin B Complex
Absorbance
1
0.8
0.6
0.4
0.2
0
220
320
420
520
620
720
Wavelength (nm)

Fig. 3

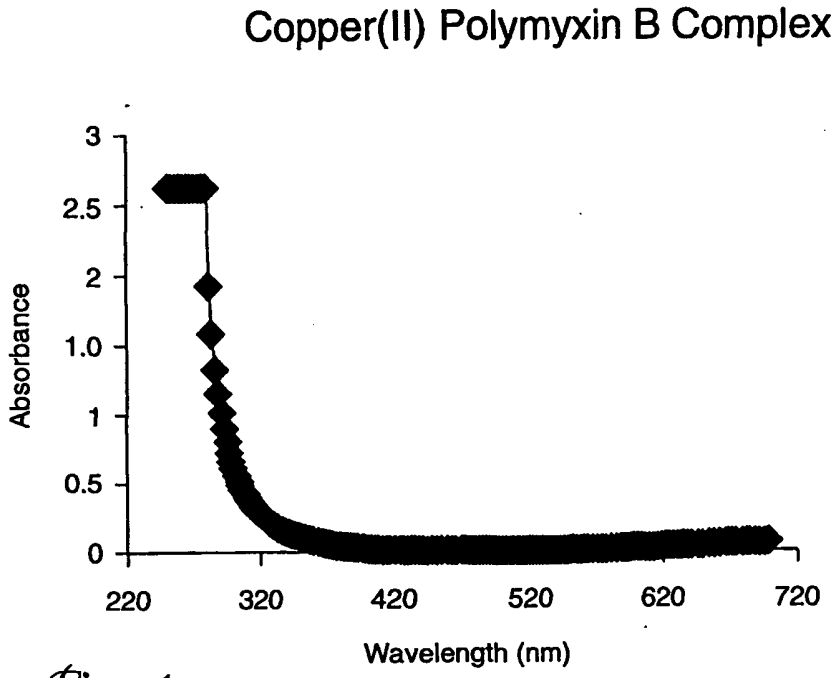

Copper(II) Polymyxin B Complex
Absorbance
3
2.5
2
1.0
1
0.5
0
220
320
420
520
620
720
Wavelength (nm)

Fig. 4

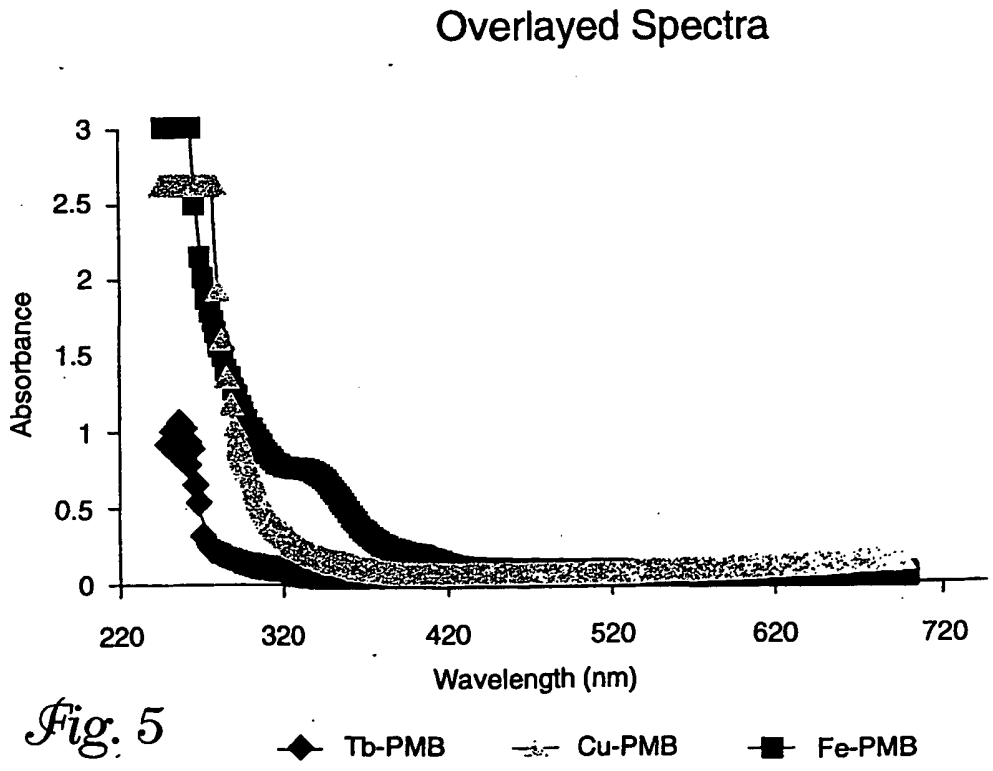

Overlayed Spectra
Absorbance
3
2.5
2
1.5
1
0.5
0
220
320
420
520
620
720
Wavelength (nm)
Tb-PMB
Cu-PMB
Fe-PMB

Fig. 5

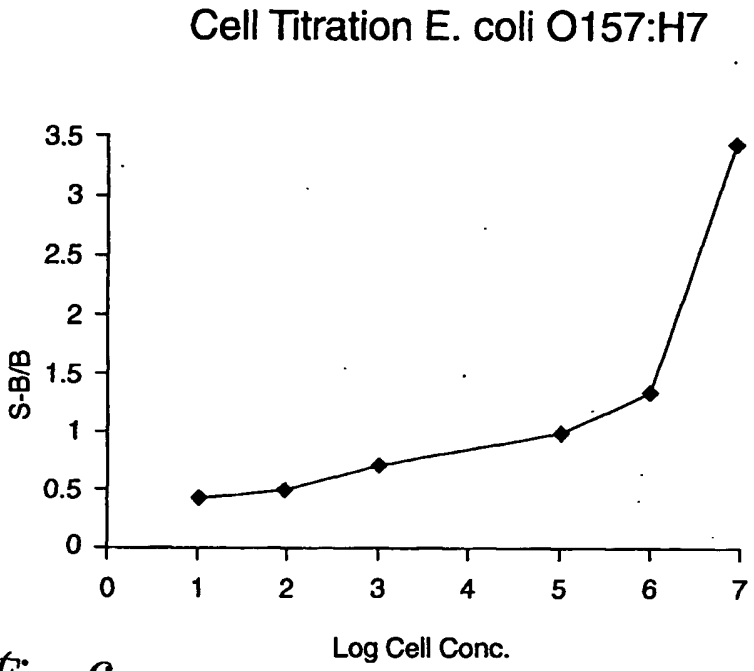

Cell Titration E. coli O157:H7
S-B/B
3.5
3
2.5
2
1.5
1
0.5
0
0 1 2 3 4 5 6 7
Log Cell Conc.

Fig. 6

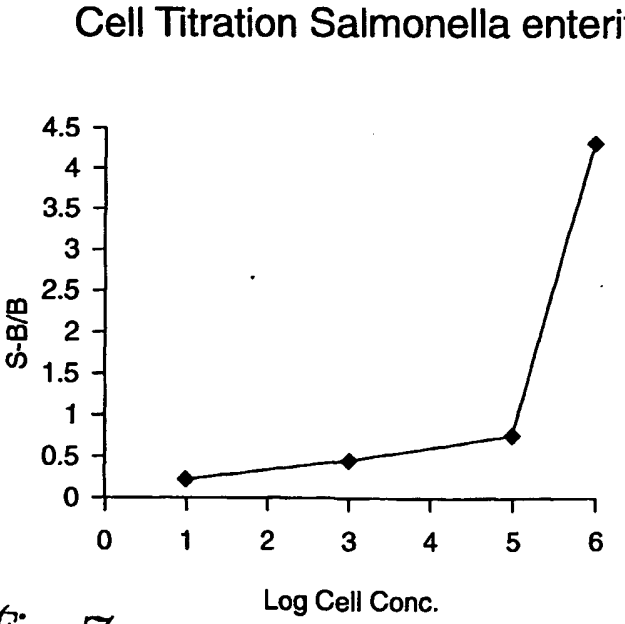

Cell Titration Salmonella enteritis
S-B/B
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0 1 2 3 4 5 6
Log Cell Conc.

Fig. 7

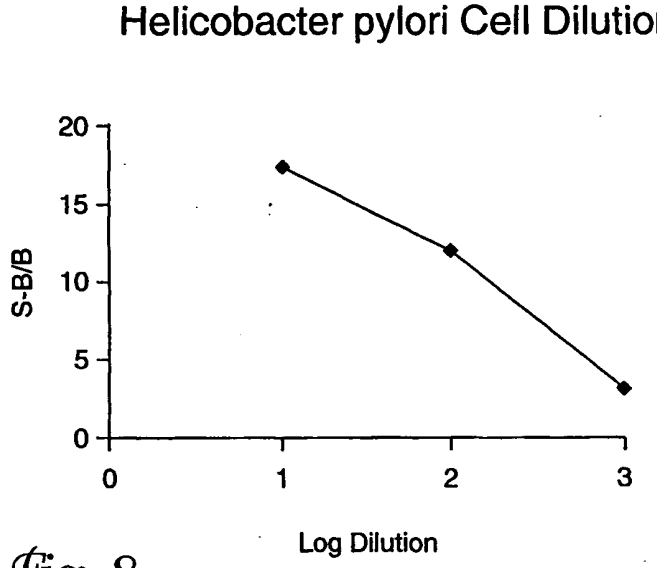

Helicobacter pylori Cell Dilution
S-B/B
20
15
10
5
0
0
1
2
3
Log Dilution

Fig. 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/28358

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	PASZKO-KOLVA ET AL. Isolation of Amoebae and Pseudomonas and Legionella spp. from Eyewash Stations. January 1991. Vol. 57, No. 1, pages 163-167, especially pages 163 and 164.	1-7, 9-13, 15 ---------- 8, 14, 71-31 and 35-41
X ------- Y	KENNEDY ET AL. Active Immobilized Antibiotics Based on Metal Hydroxides. May 1976. Vol. 9, No. 5. pages 766-770, especially pages 766 and 769-770.	1-7 and 9-10 ---------- 8, 11-15, 17-31 and 35-41

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 22 DECEMBER 2000	Date of mailing of the international search report 22 MAR 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer CHRISTOPHER LOW Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/28358

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	SLOOTMANS ET AL. Susceptability of 40 Haemophilus ducreyi Strains to 34 Antimicrobial Products. October 1983. Vol. 24, No. 4. pages 564-567, especially pages 564 and 565.	1-10 --- 11-15, 17-31 and 35-41
Y	ROST ET AL. What Do We Measure with Luminol-, Lucigenin- and Penicillin-amplified Chemiluminescence? 1. Investigations with Hydrogen Peroxide and Sodium Hypochlorite. 1998, Vol 13. pages 355-363, especially pages 355, 357-359 and 361-363.	1-15, 17-31 and 35-41
Y	US 5,750,357 A (OLSTEIN ET AL) 12 May 1998, col. 2, lines 50 to col. 9, lines 42.	1-15, 17-31 and 35-41

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/28358

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
1-15, 17-31 and 35-41

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/28358

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (7):

A61K 38/12; C07K 16/00; G01N 33/53; 33/536; C12Q 1/06; A61B 5/055; A01N 59/22

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

530/319, 317, 388.1, 388.9; 424/9.1, 9.3, 9.323, 629, 630, 646, 647, 648; 435/7.1, 7.2, 7.32, 34, 39; 436/172, 173, 542, 546; 514/9

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

530/319, 317, 388.1, 388.9; 424/9.1, 9.3, 9.323, 629, 630, 646, 647, 648; 435/7.1, 7.2, 7.32, 34, 39; 436/172, 173, 542, 546; 514/9

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CAS ONLINE, REGISTRY, MEDLINE, BIOSIS, EMBASE, WPIDS
search terms: polymyxin or polymyxin B or colistin; cyclic antibiotic, lanthamide metal or transition metal or copper or cobalt or iron or ferri or ferric or godolinium or europium or trebium or technium or metal complex; magentic resonance imagimg or MRI or chemiluminescent assay

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-15, 17-21 and 40-41, drawn to a polymyxin-metal complex, method of making, and method for detecting gram negative bacteria in a sample by using the complex thereof.

Group II, claim 16, drawn to a complex further comprising a monoclonal antibody.

Group III, claims 22-31, drawn to a method for detecting diseases in a patient by introducing a detectable complex, wherein the detecting complex comprises using magnetic resonance imaging (MRI).

Group IV, claims 32-34, drawn to a method for detecting the presence of gram negative bacteria in a patient by introducing a detectable complex, wherein the detectable complex kills or disables the gram negative bacteria.

Group V, claims 35-39, drawn to a method for treating infection in a patient by introducing a therapeutic complex.

The inventions listed as Groups I and II-V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The compound of Groups I and III-V (polymyxin-metal complex) is directed to the special technical feature, defined as a contribution over the prior art, of using polymyxin-metal complex compound as an organic "chemical tag" that comprises populations of binding agents and detectable labels as admittedly acknowledged on page 3, lines 21-24 in the instant disclosure and as taught by U.S. Patent No. 5,750,357. However, the compound of Group II is directed to a different complex in addition comprising a monoclonal antibody, and as such, is different from the compounds of Groups I and III-V because the compounds have different structures, functions and different effects.
With respect to methods of Groups I and III-V, they are directed to various methods using the same compound; i.e., Group I (claims 1-15, 17-21 and 40-41) is directed to *in vitro* assay for detecting gram negative bacteria in a sample (e.g., food sample, medical sample or biological sample); Group III (claims 22-31) is directed to *in vivo* assay for detecting diseases in a patient (e.g., by using MRI); Group IV (claims 32-34) is directed to *in vivo* assay for detecting the presence of gram negative bacteria in a patient (e.g., by killing or disabling the gram negative bacteria); and Group V (claims 35-39) is directed to a method for treating infection in a patient. Thus, the various methods using the same compound (i.e.,a polymyxin-metal complex) as recited above do not correspond to the same technical feature and are

not connected in design, operation or effect because they differ in method steps, parameters and reagents used, and as such, the methods as grouped are independent and distinct, each from the other because they represent different technical features and different inventive endeavors. Hence, the compounds used in different methods have different structures, functions and different effects. For example, Group I and Group III are directed to detect gram negative bacteria, however, Group I detects gram negative bacteria *in vitro* in a sample while Group III detects the presence of gram negative bacteria *in vivo* in a patient. Thus, the Groups require different patent and literature search and a reference teaching *in vivo* assay will not teach the *in vitro* assay. Therefore, the methods of Groups I and III-V as grouped are independent and distinct inventions which differ in methods, material make up and compositions requiring different reaction conditions. Hence, one does not require the other for ultimate use and as such is capable of separate manufacture, use and sale, and is novel and unpatentable over each other. Therefore, since the groups do not share the same special technical features, the inventions do not relate to a single inventive concept.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/28358

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

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because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
1-15, 17-31 and 35-41

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Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/28358

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (7):

A61K 38/12; C07K 16/00; G01N 33/53; 33/536; C12Q 1/06; A61B 5/055; A01N 59/22

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

530/319, 317, 388.1, 388.9; 424/9.1, 9.3, 9.323, 629, 630, 646, 647, 648; 435/7.1, 7.2, 7.32, 34, 39; 436/172, 173, 542, 546; 514/9

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

530/319, 317, 388.1, 388.9; 424/9.1, 9.3, 9.323, 629, 630, 646, 647, 648; 435/7.1, 7.2, 7.32, 34, 39; 436/172, 173, 542, 546; 514/9

B. FIELDS SEARCHED
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APS, CAS ONLINE, REGISTRY, MEDLINE, BIOSIS, EMBASE, WPIDS
search terms: polymyxin or polymyxin B or colistin; cyclic antibiotic, lanthamide metal or transition metal or copper or cobalt or iron or ferri or ferric or godolinium or europium or trebium or technium or metal complex; magentic resonance imagimg or MRI or chemiluminescent assay

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Group III, claims 22-31, drawn to a method for detecting diseases in a patient by introducing a detectable complex, wherein the detecting complex comprises using magnetic resonance imaging (MRI).

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The inventions listed as Groups I and II-V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The compound of Groups I and III-V (polymyxin-metal complex) is directed to the special technical feature, defined as a contribution over the prior art, of using polymyxin-metal complex compound as an organic "chemical tag" that comprises populations of binding agents and detectable labels as admittedly acknowledged on page 3, lines 21-24 in the instant disclosure and as taught by U.S. Patent No. 5,750,357. However, the compound of Group II is directed to a different complex in addition comprising a monoclonal antibody, and as such, is different from the compounds of Groups I and III-V because the compounds have different structures, functions and different effects.
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